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July 12, 2004

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APPLICATION NUMBER: *60/459,011*
FILING DATE: *March 31, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/09954*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

M. Tarver

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Certifying Officer

04-1-03

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03/31/03 J1049 U.S. PTO

PROVISIONAL PATENT APPLICATION TRANSMITTAL AND COVER SHEET

[Under 37 CFR 1.53(c)]

Our Docket No.: **14591**	Date: March 31, 2003
First Named Inventor: Christian Wolfrum	
Title: **Method for Inhibiting Adipogenesis and Increasing Insulin Sensitivity in Adipocytes**	
Express Mail No.: EV 040988826US	

P1036 U.S. PTO 03/31/03

ADDRESS TO: **Box Provisional Application**
Commissioner for Patents
Washington, D.C. 20231

APPLICATION ELEMENTS

1. ☒ Fee Calculation Sheet
   (Submit an original and a duplicate for fee processing)
2. ☒ Applicant claims small entity status
3. ☒ Specification Total Pages: **34**
   - ☒ Descriptive title of the invention
   - ☐ Cross References to Related Applications
   - ☒ Statement Regarding Fed. Sponsored R&D
   - ☐ Reference to Microfiche Appendix
   - ☒ Background of the Invention
   - ☒ Brief Summary of the Invention
   - ☒ Brief Description of the Drawings (if filed)
   - ☒ Detailed Description
   - ☐ Claims
   - ☒ Abstract of the Disclosure
4. ☒ Drawings (35 U.S.C. 113) Total Sheets: **24**
5. ☐ Application Data Sheet (37 C.F.R. 1.76)
6. ☐ CD-ROM or CD-R in duplicate, large table or Computer Program (Appendix)
7. ☐ Nucleotide and/or Amino Acid Sequence Submission (*If applicable, all necessary*)
   - a. ☐ Computer Readable Form (CRF)
   - b. ☐ Specification Sequence Listing on:
     - i. ☐ CD-ROM or CD-R (2 copies); or
     - ii. ☐ paper
   - c. ☐ Statement verifying identify of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 C.F.R. 3.73(b) Submission
10. ☐ Power of Attorney
11. ☒ Return Receipt Postcard
    (*Should be specifically itemized*)
12. ☐ Other

13. ☒ This invention was made by an agency of the United States Government or under a contract with an agency of the United States Government. Name of the agency: National Institutes of Health

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APPLICATION FEE TRANSMITTAL SHEET (FOR FY 2003)

Complete if Known	
Application No.	
Filing Date	March 31, 2003
First Named Inventor	Christian Wolfrum
Group Art Unit	
Examiner Name	
Atty. Docket Number	14591

METHOD OF PAYMENT (Check One)

1. ☐ The Commissioner is hereby authorized to charge indicated fees and credit any over payments to:
   Deposit Account No.: 50-2054
   Deposit Account Name: DORSEY & WHITNEY LLP
   ☒ Charge any additional fee required under 37 C.F.R. 1.16 and 1.17
   ☐ Applicant claims small entity status (see 37 C.F.R. 1.27)
2. ☒ Check Enclosed

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description	Fee Paid
101	750	201	375	☒ Utility Filing Fee	
106	330	206	165	☐ Design Filing Fee	
108	750	208	375	☐ Reissue Filing Fee	
114	160	214	80	☒ Prov. Filing Fee	80.00
				Subtotal (1)	80.00

2. EXTRA CLAIM FEES

	Number Claims		Prior		Extra		Fee from Below		Fee Paid
Total		-	20	=		x	9/18	=	
Indep.		-	3	=		x	42/84	=	
Multiple Dependent Claims						x	140/280	=	
							Subtotal (2)		

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	84	202	42	Independent claims in excess of 3
104	280	204	140	Multiple dependent Claim
109	84	209	42	Reissue independent claims over original patent
110	18	210	9	Reissue claims in excess of 20 and over original patent

FEE CALCULATION (Continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Fee ($)	Small Entity Fee Code	Fee ($)	Fee Description	Fee paid
105	130	205	65	Surcharge - Late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
115	110	215	55	Extension for reply within first month	
116	410	216	205	Extension for reply within second month	
117	930	217	465	Extension for reply within third month	
118	1,450	218	725	Extension for reply within fourth month	
128	1,970	228	985	Extension for reply within fifth month	
120	320	220	160	Filing a brief in support of an appeal	
121	280	221	140	Request for oral hearing	
138	110	238	55	Terminal Disclaimer Fee	
140	110	240	55	Petition to revive – unavoidable	
141	1,280	241	640	Petition to revive – unintentional	
142	1,300	242	650	Utility/Reissue issue fee (inc. advance copies)	
143	470	243	235	Design issue fee (inc. advance copies)	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	300	195	300	Publication fee for early, voluntary, or normal publication	
196	300	196	300	Publication fee for republication	
126	180	126	180	Submission of IDS	
581	40	81	40	Recording each patent assignment per property (times number of properties)	
179	750	279	375	Request for Continued Examination (RCE)	
				Other fee (specify)	
				Subtotal (3)	
				Total Amount of Payment:	80.00

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Signature: Janet M. MacLeod		Date: March 31, 2003

WE, CHRISTIAN WOLFRUM, DAVID Q. SHIH, SATORU KUWAJIMA and MARKUS STOFFEL, citizens of Germany, the United States, Japan and Germany, respectively, and residents of the United States, have invented certain new and useful improvement in :

METHOD FOR INHIBITING ADIPOGENESIS AND INCREASING INSULIN SENSITIVITY IN ADIPOCYTES

of which the following is a

SPECIFICATION

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

[0001] This invention was made with government support under National Institutes of Health Grant RO1 DK55033-04 and Medical Scientists Training Program Grant GM07739. The government may have certain rights in the invention.

BACKGROUND OF THE INVENTION

[0002] Obesity results from the expansion of white adipose tissue (WAT) by the recruitment of adipocyte precursor cells, and is a major cause of insulin resistance and diabetes. The process of adipocyte differentiation is the focus of extensive research, and a cascade of transcription factors that are responsible for this conversion have been identified. Rosen et al. (2000) Annv. Rev. Cell. Dev. Biol. 16:145-171. In addition, a number of factors that inhibit adipogenesis have been identified including the extracellular signaling molecules interleukin-1, tumor necrosis factor α (TNFα) and the cell surface protein preadipocyte factor-1 (Pref-1). Ohsumi et al. (1994) Endocrinol. 135:2279-2282; Smas et al. (1993) Cell 73:725-734.

[0003] Hepatocyte nuclear factors-3 (Foxa1-3) are winged forkhead transcription factors that are required for normal metabolism by regulating expression of genes that are essential for liver and pancreatic islet function. The forkhead transcription factor Foxa-2 (Hnf-3β) is related to Foxa-1, a transcriptional activator of the glucagon gene, and Foxa-3, a transcriptional regulator of the gluconeogenic enzyme phosphoenopyruvate carboxykinase (Pepck). Kaestner et al. (1999) Genes Dev. 13:495-504; Shih et al. (1999) Proc. Natl. Acad. Sci. USA 96:10152-10157; Kaestner et al. (1998) Mol. Cell. Biol. 18:4245-4251. It has been surprisingly discovered in accordance with the present invention that Foxa-2 plays a crucial role in the regulation of adipocyte differentiation and metabolism.

[0004] The nuclear hormone receptor farnesoid X receptor (Fxr) is a bile acid-activated receptor that regulates hepatic biosynthesis of bile acids from cholesterol. Fxr positively regulates the expression of several genes involved in lipoprotein metabolism, and thus contributes to the maintenance of proper plasma cholesterol and triglyceride levels. In accordance with the present invention, it has been found that Fxr also plays an important role in adipocyte differentiation and metabolism.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention provides a method for inhibiting adipogenesis comprising contacting a cell with an agent that increases levels of Foxa-2 mRNA and/or protein.

[0006] The present invention further provides a method for inhibiting adipogenesis comprising contacting a cell with an agent that increases the levels of Fxr mRNA and/or protein, or an agent that activates Fxr.

[0007] The present invention further provides a method of treating obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus

comprising administering to a subject in need of such treatment a composition comprising an agent that increases Foxa-2.

[0008] The present invention further provides a method of treating obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus comprising administering to a subject in need of such treatment a composition comprising an agent that increases the levels of Fxr mRNA and/or protein, or an agent that activates Fxr.

[0009] In another embodiment, the present invention provides methods of identifying agents that increase Foxa-2, agents that activate Fxr and agents that increase Fxr. Such agents are useful for the treatment of obesity, metabolic syndrome, and non-insulin dependent diabetes mellitus.

[0010] Agents that increase Foxa-2, agents that activate Fxr and agents that increase Fxr and compositions comprising such agents are also provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Figs. 1a-f demonstrate the expression of Foxa-2 in adipose tissue. Fig. 1a is a Western blot of liver and adipose tissue extracts analyzed for Foxa-2 expression. Fig. 1b is a Northern blot of visceral and subcutaneous fat from wt and ob/ob mice analyzed for Foxa-2 expression. Fig. 1c is a Western blot of preadipocyte (Pre) and adipocyte (Ad) protein extracts. Figs. 1d-f are images from confocal image immunostaining of visceral fat from an ob/ob mouse.

[0012] Figs. 2a-c demonstrate that Foxa-2 expression is induced by insulin. Fig. 2a is a graph depicting the correlation of Foxa-2 and Foxc-2 mRNA expression with plasma insulin concentration in various mouse models. Figs. 2b and 2c demonstrate the effect of insulin and other factors on Foxa-2 expression in primary adipocytes from wt (Fig. 2b) and ob/ob (Fig. 2c) mice.

[0013] Figs. 3a-e demonstrate that Foxa-2 inhibits adipocyte differentiation in 3T3-L1 cells.

[0014] Figs. 4a-e show that Foxa-2 regulates genes involved in glucose uptake, glycolysis, lipolysis and energy dissipation.

[0015] Figs. 5a-n depict the development of diet-induced obesity and metabolic analysis of primary adipocytes of Foxa-$2^{+/-}$ and wildtype littermates.

[0016] Fig. 6, demonstrates the expression of Fxr in embryoid bodies deficient for Foxa-2.

[0017] Fig. 7 is a graph depicting transactivation of murine and human Fxr by members of the hepatocyte nuclear factor (HNF) family.

[0018] Fig. 8 depicts an electrophoretic mobility shift analysis of the Foxa-binding site in the Fxr-1 promoter.

[0019] Figs. 9a and b depict the de novo expression of Fxr-1 in adipose tissue of ob/ob mice, and primary adipocytes of lean mice stimulated with insulin.

DETAILED DESCRIPTION OF THE INVENTION

[0020] In accordance with the present invention it has been discovered that the winged forkhead transcription factor Foxa-2 (previously designated hepatocyte nuclear factor-3β, HNF-3β) is induced de novo in visceral and subcutaneous fat of genetic and diet-induced mammalian models of obesity. Foxa-2 expression can be induced by insulin in primary adipocytes, and Foxa-2 levels in fat positively correlate with fasting serum insulin concentrations of hyperinsulinemic animals. The expression of Foxa-2 inhibits adipocyte differentiation in vitro and activates genes involved in glucose and fat metabolism. Diet-induced obese mice with haplosufficiency in Foxa-2 develop increased adiposity compared to wildtype littermates, and adipocytes of these mice exhibit defects in glucose uptake and metabolism. These discoveries show that Foxa-2 is an insulin-regulated gene that inhibits adipocyte differentiation and

plays a crucial role as a physiological regulator of adipocyte differentiation and metabolism. Induction of Foxa-2 expression stimulates a protective mechanism that counteracts excessive actions of insulin in preadipocytes and enhances insultin sensitivity in mature adipocytes.

[0021] Accordingly, the present invention provides a method for inhibiting adipogenesis comprising contacting a cell capable of adipogenesis with an agent that increases levels of Foxa-2 mRNA and/or protein. In a preferred embodiment the agent induces expression of Foxa-2. The invention further provides a method of treating obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus comprising administering to a subject in need of such treatment a composition comprising an agent that increase Foxa-2. The term "Foxa-2" as used herein refers to Foxa-2 from any species. In a preferred embodiment Foxa-2 is mammalian Foxa-2. In a more preferred embodiment Foxa-2 is human Foxa-2.

[0022] Agents that induce expression of Foxa-2 can be identified by a screening method which provides another embodiment of a present invention. The method of screening for compounds that induce Foxa-2 expression comprises contacting a plurality of cells that contain a Foxa-2 promoter operably linked to a coding sequence for Foxa-2 with a candidate agent, assaying for Foxa-2 expression in the presence and absence of the candidate agent, and comparing Foxa-2 expression in the presence and absence of the candidate agent, whereby an increase in Foxa-2 expression in the presence of the candidate agent is indicative of the identification of an agent that increases Foxa-2 expression.

[0023] In a preferred embodiment of the present method, the cells are mammalian cells. More preferably the cells are human. The cells may be cells that comprise the Foxa-2 gene but do not express Foxa-2 under normal culture conditions. Such cells include preadipocytes and adipocytes. In a preferred embodiment the cells are 3T3-L1 cells or primary preadipocytes or adipocytes of

lean subjects. The cells may be isolated and cultured by conventional methods, or obtained commercially. Human preadipocytes and adipocytes are commercially available.

[0024] The cells may also be cells that have been engineered to contain a construct comprising the Foxa-2 promoter operably linked to the coding sequence for Foxa-2. Mammalian Foxa-2 genes are known in the art, and the promoters and coding regions have been sequenced and characterized. See, e.g. Kaestner (2000) TEM 11: 281-283. Different isoforms of Foxa-2 exist, and are derived from alternative first exons and differential splicing at the 5' end of the gene. Sasaki et al. (1994) Cell 76: 103-115. It has been determined by 5'-RACE analysis that the adipocyte-specific Foxa-2 isoform is encoded by the L1 transcript. In a preferred embodiment of the present invention, the coding sequence encodes the adipocyte-specific Foxa-2 isoform. In a preferred embodiment the Foxa-2 promoter for adipocyte expression is located upstream of exon L1. The mouse Foxa-2 promoter is known in the art and disclosed, e.g. at NCBI Genome database entry L25669 and by Sasaki et al. (1994) Cell 76: 103-115. The human Foxa-2 promoter is known in the art and disclosed, e.g. at NCBI accession number AL121722. Those of ordinary skill in the art can identify the promoter, as well as fragments, modifications and variants thereof that are effective to direct expression of Foxa-2 in adipocytes. Foxa-2 coding regions are also known in the art. In a preferred embodiment, the Foxa-2 coding region is the mouse sequence disclosed at NCBI Genome database entry U04197 or the human sequence disclosed at NCBI entry NM153675 and Yamada et al. (2000) Diabetologia 43: 121-124. Those of ordinary skill in the art can identify fragments, variants and modifications of these sequences that retain the ability to encode a Foxa-2 polypeptide having the function of inhibiting adipocyte differentiation and increasing insulin sensitivity in adipocytes. The term

"operably linked" is understood to mean that the promoter directs the expression of protein encoded by the coding sequence.

[0025] The construct can be introduced into a host cell by methods known in the art. The construct is preferably provided within an expression vector that is suitable for introduction into a host cell, and that contains nucleic acid sequences that control expression. Expression vectors are well-known in the art, and may be constructed by conventional methods. A starting vector may be obtained commercially and modified to include the present construct. In a preferred embodiment, the vector is the pGL2-Enhancer Vector (Promega).

[0026] The vector may be introduced into a host cell by methods well-known in the art. Transformation of a host cell may be accomplished, for example, by transfection, infection, electroporation, microinjection, and other well-known techniques set forth in laboratory manuals including Sambrook et al. (2001) Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, incorporated herein by reference. Host cell lines are well-known in the art and are commercially available. Cell lines stably transformed with the vector of the invention are preferred. In a preferred embodiment, the host cell is a mammalian preadipocyte or adipocyte. More preferably, the host cell is a human preadipocyte or adipocyte.

[0027] Host cells comprising the Foxa-2 promoter and coding sequence are cultured under standard conditions known in the art and contacted with a candidate agent.

[0028] Candidate agents include any chemical compound, and may be naturally occurring or synthetic. Combinatorial libraries of candidate agents may be used. In a preferred embodiment, well-known automated methods of high throughput screening are used to assay candidate agents. Agents that can be transported into adipocytes or formulated for transport into adipocytes are preferred.

[0029] Foxa-2 expression may be assayed by detecting Foxa-2 mRNA by conventional methods, for example by Northern blotting using Foxa-2 specific probes or quantitative polymerase chain reaction (PCR) using Foxa-2 specific primers. Foxa-2 expression may also be assayed by detecting Foxa-2 protein, for example by Western blotting or immunohistochemistry using anti-Foxa-2 antibodies. Such antibodies may be generated by methods known in the art or obtained commercially.

[0030] An increase in Foxa-2 expression in the presence of the candidate agent relative to expression in the absence of the agent is defined herein as an increase that is detectable by any of the foregoing methods. Agents identified by the screening method of the invention may be used as potential therapeutics or may serve as lead compounds for the development of therapeutics.

[0031] The present invention also provides a method of screening for compounds that induce Foxa-2 expression comprising contacting a plurality of cells that contain a Foxa-2 promoter operably linked to the coding sequence of a reporter gene with a candidate agent, assaying for the expression of the reporter in the presence and absence of the candidate agent, and comparing expression of the reporter in the presence and absence of the candidate agent, whereby an increase in the expression of the reporter in the presence of the candidate agent is indicative of an agent that increases Foxa-2 expression.

[0032] The method is performed as described hereinabove except that the Foxa-2 coding sequence is replaced by a reporter sequence, and detection of expression of Foxa-2 is replaced by detection of expression of the reporter.

[0033] Reporter genes that encode easily assayable reporter proteins are well-known in the art. In general, a reporter gene is a gene which is not normally present or expressed in the host cell, and which expresses a protein having an easily detectable property. Preferred reporter genes include the chloramphenicol acetyl transferase (cat) gene, the beta-galactosidase (gal) gene, the beta-

glucuronidase (gus) gene, the green fluorescence protein (GFP) gene, and the luciferase (luc) gene. The methods of detection of these reporters are well-known in the art, and are dictated by the nature of the reporter. For example, beta-galactosidase hydrolyzes galactosides to yield detectable colored products.

[0034] In a preferred embodiment of these methods, the host cell is a 3T3-L1 cell that has been stably transformed with a construct comprising the Foxa-2 promoter operably linked to the luciferase gene.

[0035] Agents identified by the foregoing screening methods are useful for inhibiting adipogenesis and for treating obesity, metabolic syndrome, and diabetes. The present invention provides compositions comprising such agents. The compositions may further comprise a diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant, and are preferably formulated for transport into adipocytes.

[0036] The formulation of pharmaceutical compositions is generally known in the art and reference can conveniently be made to Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa. Formulations for use in present invention must be stable under the conditions of manufacture and storage and must also be preserved against the contaminating action of microorganisms such as bacteria and fungi. Prevention against microorganism contamination can be achieved through the addition of various antibacterial and antifungal agents.

[0037] The pharmaceutical forms of the present agents suitable for administration include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. Typical carriers include a solvent or dispersion medium containing, for example, water buffered aqueous solutions (i.e., biocompatible

buffers), ethanol, polyols such as glycerol, propylene glycol, polyethylene glycol, suitable mixtures thereof, surfactants, or vegetable oils.

[0038] Sterilization can be accomplished by any art-recognized technique, including but not limited to filtration or addition of antibacterial or antifungal agents, for example, paraben, chlorobutanol, phenol, sorbic acid or thimerosal. Further, isotonic agents such as sugars or sodium chloride may be incorporated in the subject compositions.

[0039] Production of sterile injectable solutions containing the subject agents is accomplished by incorporating these compounds in the required amount in the appropriate solvent with various ingredients enumerated above, as required, followed by sterilization, preferably filter sterilization. To obtain a sterile powder, the above solutions are vacuum-dried or freeze-dried as necessary.

[0040] The subject agents are thus compounded for convenient and effective administration in pharmaceutically effective amounts with a suitable pharmaceutically acceptable carrier and/or diluent in a therapeutically effective dose.

[0041] The methods of inhibiting adipogenesis and enhancing insulin sensitivity may also be accomplished by contacting a cell with Foxa-2 protein or a vector capable of expressing Foxa-2 protein in preadipocytes and/or adiopocytes. The vector may comprise a construct having a constitutive promoter operably linked to a nucleic acid encoding Foxa-2. The method of treating obesity, metabolic syndrome or diabetes may be accomplished by administering a composition comprising Foxa-2 protein or a vector capable of expressing Foxa-2 protein.

[0042] It has further been discovered in accordance with the present invention that adipogenesis may be inhibited by increasing or activating the nuclear hormone receptor Fxr. In particular, it has been discovered that Fxr is expressed de novo in adipocytes of obese (hyperinsulinemic) mice, and that Fxr

expression can be induced by culturing primary adipocytes of lean mice in the presence of insulin. Increased levels of Fxr, or activation of Fxr by endogenous or synthetic ligands, is likely to lead to induction of genes that enhance insulin sensitivity in adipocytes. The term "Fxr" as used herein refers to Fxr from any species. In a preferred embodiment, Fxr is mammalian Fxr. In a more preferred embodiment, Fxr is human Fxr.

[0043] Accordingly, the present invention provides a method for inhibiting adipogenesis comprising contacting a cell capable of adipogenesis with an agent that increases levels of Fxr mRNA and/or protein, or an agent that activates Fxr. The invention further provides a method of treating obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus comprising administering to a subject in need of such treatment a composition comprising an agent that increases or activates Fxr.

[0044] A method of screening for agents that increase Fxr expression comprises contacting a plurality of cells that contain an Fxr promoter operably linked to a coding sequence for Fxr with a candidate agent, assaying for Fxr expression in the presence and absence of the candidate agent, and comparing Fxr expression in the presence and absence of the candidate agent, whereby an increase in Fxr expression in the presence of the candidate agent is indicative of the identification of an agent that increases Fxr expression.

[0045] In a preferred embodiment, the cells are mammalian cells. More preferably, the cells are human. The cells may be cells that comprise the Fxr gene but do not express Fxr under normal culture conditions. Such cells include preadipocytes and adipocytes. In a preferred embodiment the cells are 3T3-L1 cells.

[0046] The cells may also be cells that have been engineered to contain a construct comprising the Fxr promoter operably linked to the coding sequence of Fxr. Mammalian Fxr genes are known in the art, and the promoters and coding

sequences have been sequenced and characterized. See, e.g. Chiang (2002) Endocrine Reviews 23:443-463 and U.S. Patent Application Publication 2003/0003520A1. In a preferred embodiment, the Fxr promoter is contained within a 1245 base pair fragment upstream of the coding sequence (ATG) of the human Fxr gene (NCBI nucleotide database NT-009743) and the Fxr coding sequence is provided at NCBI nucleotide database NM-005123 and disclosed by Forman et al. (1995) Cell 81: 687-693.

[0047] The constructs may be introduced into host cells as described above. Candidate agents are defined and screening may be performed as described above.

[0048] Fxr expression may be assayed by detecting Fxr mRNA by conventional methods, for example by Northern blotting using Fxr-specific probes, or by quantitative PCR using Fxr-specific primers. Fxr expression may also be assayed by detecting Fxr protein, for example by Western blotting or immunohistochemistry using anti-Fxr antibodies. Anti-Fxr antibodies may be generated by conventional methods.

[0049] An increase in Fxr expression is defined as an increase that is detectable by any of the foregoing methods.

[0050] The method of detecting agents that increase Fxr expression may be modified as described above to substitute the coding sequence of a reporter gene for the Fxr coding sequence, and assaying for expression for the reporter.

[0051] In a preferred embodiment of this method, the host cell is a 3T3-L1 cell that has been stably transformed with a construct comprising the Fxr promoter operably linked to the luciferase gene.

[0052] The present invention further provides a method for screening for agents that activate Fxr in adipose tissue. The method comprises contacting a plurality of cells that contain Fxr with a candidate agent, assaying for activation of Fxr in the presence and absence of a candidate agent, and comparing Fxr

activation in the presence and absence of the candidate agent, whereby an increase in activation in the presence of the agent is indicative of the identification of an agent that activates Fxr. Activation of Fxr may be assessed by measuring reporter gene activity in cells that are transfected with a vector containing the Fxr promoter upstream of a reporter gene, e.g. the luciferase gene. Activation of Fxr may also be measured by measuring the increased expression of known target genes of Fxr, such as the small heterodiner partner (Shp) gene. Known activators of Fxr include naturally occurring agents such as bile acids (chenodeoxycholic acid (CDCA) and cholic acid (CA)), farnesol, juvenile hormone III, all-trans-retinoic acid and synthetic compounds such as GW4064 (Glaxo Smith Kline).

[0053] Agents that increase or activate Fxr in adipose tissue are useful for inhibiting adipogenesis and for the treatment of obesity, metabolic syndrome, and diabetes. Agents identified by the foregoing methods may be used as potential therapeutics or may serve as lead compounds for the development of therapeutics.

[0054] The present invention further provides compositions comprising agents that increase or activate Fxr. Such compositions may contain additional components, and may be formulated and delivered as described herinabove.

[0055] All references cited herein are incorporated herein in their entirety.

[0056] The following nonlimiting examples serve to further illustrate the present invention.

Example 1

Materials and Methods

[0057] The following materials and methods were used in subsequent examples.

Plasmids

[0058] The hPREF-1 promoter (681 bp) was cloned from a human lambda Fix II™ library (Stratagene). The murine m-Pk (989 bp), [HK-2] (771 bp), Lpl (1011 bp), Hsl (599 bp) and Ucp-2 (877 bp) promoters were amplified using site specific primers and genomic DNA as template and cloned into the pGL2-Enhancer vector (Promega). The sequences of all the clones were confirmed by dideoxynucleotide sequencing.

Animals and metabolic cages

[0059] All animal models were maintained in C57B1/6J background and maintained on a 12 hours light/dark cycle in a pathogen-free animal facility. Oxygen consumption, CO_2 and heat production and food and water intake were simultaneously determined for 4 mice per experiment in an Oxymax metabolic chamber system (Columbus Instruments, Columbus, Ohio). Individual mice (15 weeks) were placed in a chamber with an airflow of 0.6 L/min and one reading per mouse was taken at 4-minute intervals over 24 hours. Resting metabolic parameters were determined by integrating values at periods of no activity.

Electrophoretic mobility shift assay

[0060] Nuclear extracts were prepared as described by Stuempfle et al. (1996) Biotechniques 21:48-50, with minor modifications. Visceral fat from wt and ob/ob animals was washed in pre-chilled phosphate buffered saline. (PBS) supplemented with protease inhibitor cocktail (Roche) and homogenized in sucrose buffer (20 mM Hepes pH 7.9, 25 mM KC1, 2 M sucrose, 20% (v/v) glycerol, 1mM EDTA, protease inhibitor cocktail) using a dounce homogenizer. The homogenized tissue was centrifuged over a sucrose buffer cushion (100,000g; 40 mm), and the nuclei were resuspended in lysis buffer (20 mM Hepes pH 7.9, 420 mM NaC1, 1.5 mM $MgC1_2$, 0.2 mM EDTA, protease inhibitor cocktail, 25% (v/v) glycerol). After 30 minutes of incubation, nuclear extracts were centrifuged

(45,000g, 30 mm) and the supernatant was snap frozen in liquid nitrogen. Protein content was measured by BCA-test.

[0061] Nuclear extracts from ob/ob fat (20 µg) were incubated with ^{32}P-labeled double-stranded oligonucleotide probes with either the wt or a mutated putative Foxa binding site from the PREF-1-, Lpl- and Ucp2-promoter. The reaction was performed in a mixture containing Hepes buffer (20 mM, pH 7.9), KCl (40 mM), $MgCl_2$ (1 mM), EGTA (0.1 mM), DTT (0.5 mM), 4% Ficoll and poly(dIdC) at room temperature for 10 minutes. Competition analysis was performed by incubating the cellular extracts and the probe with the non-labeled oligonucleotide. Supershift analysis was carried out by incubating the nuclear extracts with either anti-Foxa-1 or anti-Foxa-2 antibody disclosed by Ruiz I. Altaba et al. (1993) Mech. Dev. 44:91-108. The reaction mixture was loaded on a 6% non-denaturing polyacrylamide gel in TBE buffer (0.023 M Tris-borate, 0.5 mM EDTA) and run at 4°C. Bands were visualized by autoradiography. The sequence of the binding sites are PREF- 1: 5' -GTGTGTAATTATGTGCTTAG-3', Lpl: 5'CTTATTTGCATATTTCCAGT-3', Ucp-2: 5' -CAGGTTGCCTGTTTGTTTTC-3'.

Cell culture

[0062] 3T3-L1 cells were maintained in DMEM with 4.5 g/l glucose, 10% fetal calf serum, 2 mM glutamine, 1 mM pyruvate and penicillin/streptomycin (Life Technologies, Inc.) in a humidified incubator at 5% CO_2. Cells were subcultured at a split ratio of 1:4. Adipocyte differentiation was induced as described by treating with 1 µM dexamethasone (Sigma) and 0.5 mM MIX (Sigma) for 8 days in the presence or absence of insulin (5 nM) (3). Incorporation of lipids was visualized by staining with Oil Red 0 (Sigma).

Transactivation assay

[0063] 3T3-Ll cells were grown to 60-70% confluence and subsequently transfected with the reporter genes (0.5 μg), pCMV-β-Gal as internal reference (0.5 μg) and the expression vectors for Foxa-1 and Foxa-2 (0.5 μg) or pcDNA3 alone as control by use of the transfection reagent Fugene6 according to manufacturer's protocol (Roche). Cells were grown for an additional 48 hours after transfection. Luciferase activity was measured using the Luciferase Detection System following the manufacturer's protocol (Promega). Luciferase was normalized for transfection efficiency by the corresponding β-galactosidase activity as described by Alam et al. (1990) Anal. Biochem. 188:245-254.

Generation of the stable cell lines

[0064] 3T3-Ll cells were plated at a density of 20,000 cells/cm^2 and transfected with the expression vector pcDNA3-Foxa-1, -Foxa-2, -Pref-1, using Fugene6 (Roche) as transfection reagent. The transfected cells were selected in 350 μg/ml of G418 (Life Technologies, Inc.) and approximately two hundred G418 resistant clones were pooled and expanded in selection medium. Expression of the stably transfected gene was confirmed by RT-PCR.

Reverse Transcriptase-PCR

[0065] Total RNA was extracted from cells and EBs using Trizol following manufacturer's instructions (Life Technologies, Inc.). Contaminating genomic DNA was removed by treating with 5 u of RNase-free DNase-I (Roche Molecular Biochemicals)/10 μg of RNA. cDNA was synthesized using moloney leukemia virus reverse transcriptase with dNTPs and random hexamer primers (Stratagene). The cDNAs provided templates for polymerase chain reactions (PCRs) using specific primers at annealing temperatures ranging between 60 and 65°C in the presence of dNTPs, [α^{-32}P]dCTP, and Taq DNA polymerase. PCR

synthesis for each primer pair was quantified at 15, 20, 25, and 30 cycles in a test reaction to ensure that the quantitative PCR amplification was in the linear range.

Northern blot analysis

[0066] Specific DNA probes were generated using the Highprime DNA labeling kit following the manufacturer's instructions (Roche). Total RNA fat tissue was prepared using Trizol as described by the manufacturer (Life Technologies, Inc.) and separated (30 μg per lane) on a 1% agarose gel containing 5% formaldehyde. After blotting onto a positively charged nylon membrane (Schleicher&Schuell), the blot was hybridized at 42°C with the respective probe using Hybrisol hybridization buffer (Intergen).

Western blot analysis

[0067] Cytosolic protein extracts were separated by SDS-PAGE (13.5%) and transferred onto a nitrocellulose membrane (Schleicher&Schuell) by electroblotting. A-FABP was detected with anti-human aP2-antiserum (1:500) (F. Spener, Muenster, Germany) and goat anti-rabbit IgGs conjugated to HRP (1:10,000) in TBS supplemented with 5 % nonfat dry milk. Foxa-2 was detected with anti-Foxa-2 antiserum (28)(1:1000) and goat anti-rabbit IgG conjugated to HRP (1:10,000). All antibodies were dissolved in 5% milk in TBS with 0.5% Tween-20. The blots were washed three times for 15 minutes between incubations. Membranes were incubated with primary antibodies overnight at 4°C. Incubations containing the secondary antibody were performed at room temperature for 1 hour. For visualization, the Renaissance Chemiluminescence Substrate (NEN, MA) was used.

Adipocyte isolation and metabolic studies

[0068] Primary adipocytes were isolated as described by Rodbell (1964) J. Biol. Chem. 239:375-380. For the assessment of lipogenesis, lipolysis and glucose metabolism of a 10% isolated fat cell suspension at 5 mM glucose was used. Glucose transport of isolated adipocytes was measured by incubation for 30 minutes with 3 μM U-[^{14}C]glucose with or without insulin stimulation as described by Black et al. (1995) J. Cell. Biochem. 58:435-463. The reaction was stopped by spinning through dinonyl phtalathe oil and the radioactivity quantified by scintillation counting.

[0069] Glucose incorporated into triglycerides, lactate and CO_2 was measured after 2 hours incubation with 3 μM U-[^{14}C]glucose in the absence or presence of 100 nM insulin as described by Tozzo et al. (1995) Am. J. Physiol. 268:E956-E964. Fatty acid de novo synthesis was analyzed by saponification of total lipids as described by Shakir et al. (1978) J. Lipid Res. 19:433-442 and quantification of radioactive label into fatty acids. Incorporation into lipid glycerol was calculated by subtracting fatty acid radioactive label from total lipid radioactive label.

[0070] To quantify lipolysis, isolated adipocytes (200 μl of a 10% isolated fat cell suspension) were incubated in the presence of adenosine deaminase and 10 μM PIA (N6[R-(-)- 1 -methyl-2-phenyl]adenosine), with or without 100 μM isoproterenol to produce maximal increase of lipolysis for 30 minutes in the presence or absence of insulin. Glycerol content of the incubation medium was determined using a radiometric assay as described by Susulic et al. (1995) J. Biol. Chem. 270:29483-20402.

Statistical Analysis

[0071] Results are given as mean ± SD. Statistical analyses were performed by using a Student's t-test, and the null hypothesis was rejected at the 0.05 level. Linear regression was calculated using Origin (Microcal).

Example 2

Expression of Foxa-2 (Hnf-3β) in Adipose Tissue

[0072] To identify genes that play a role in adipocyte differentiation and obesity, gene expression in adipose tissue of wildtype (wt) and obese (ob/ob) mice and monogenic (db/db) and polygenic (NZO) animal models of obesity was compared. Liver cell extracts from wt mice and adipocyte extracts from wt, ob/ob, db/db and NZO mice were separated by SDS-PAGE and analyzed by Western blotting for Foxa-2 expression. TATA binding protein (Tbp) expression was measured as a control for loading.

[0073] As shown in Fig. 1a, the forkhead transcription factor Foxa-2 (Hnf-3β) was undetectable in fat tissue of wt mice, and was expressed in adipose tissue of obese mice. Expression of Foxa-2 was also found in the monogenic (db/db) and polygenic (NZO) models of obesity and in fat tissue of wt mice in which obesity was induced with a high fat diet (Fig. 2a, C57,HF).

[0074] Of the Fox genes, only Foxa-2 expression was detected in adipose tissue of ob/ob mice.

[0075] Visceral and subcutaneous fat RNAs from wt and ob/ob mice were analyzed for Foxa-2 expression by Northern blotting. The membrane was rehybridized with a probe for cyclophilin as a loading control. As shown in Fig. 1b, Foxa-2 was expressed in visceral and subcutaneous fat of ob/ob mice, but was enriched in visceral fat depots. De novo expression of Foxa-2 was specific for adipocytes and was not observed in other insulin-sensitive tissues such as muscle.

[0076] Preadipocyte (Pre) and adipocyte (Ad) protein extracts from ob/ob mice were separated by SDS-PAGE and analyzed by Western blotting for Foxa-2 and aP2 expression. As shown in Fig. 1c, both the adipocycte fraction and the

stromal fraction of adipocytes containing preadipocyctes expressed the Foxa-2 protein.

[0077] Confocal image immunostaining of visceral fat from an ob/ob mouse using anti-Foxa-2 antibodies (Fig. 1d) and TO-PRO-3 molecular probes for nuclear staining (Fig. 1e) was performed. Superimposed images are shown in Fig. 1f. As shown in Figs. 1d-1f, Foxa-2 protein was detected in the nuclei and cytoplasm of adipocytes of obese animals.

Example 3

Foxa-2 expression in adipocytes correlates with insulin levels

[0078] Foxa-2 and Foxc-2 mRNA expression in fat of various mouse models was quantified by counting the radioactive product obtained by RT-PCR and normalizing it to the Hprt RT-PCR product. All values were calculated relative to the highest mRNA expression and correlated to plasma insulin concentration.

[0079] As shown in Fig. 2a, a striking correlation of Foxa-2 expression in fat tissue of obese mice and fasting plasma insulin levels was observed ($R=0.99$, $p<0.0001$). The correlation between plasma insulin levels and adipocyte Foxa-2 expression was markedly stronger than with Foxc-2. Foxc-2 is a forkhead transcription factor that is induced by insulin in fat cells. Cederberg et al. (2001) Cell 106:563-573.

[0080] No statistically significant linear correlation was detected between adipocyte Foxa-2 mRNA levels and TNF-α, leptin, triglycerides (TG), free fatty acids (FFA) or fasting blood glucose concentrations. Foxa-2 expression of ob/ob mice that were starved for 5 days decreased 2.6±0.3-fold ($p<0.001$) and was accompanied by lowered plasma insulin levels (19±0.7 vs. 0.7±0.1 ng/ml). The adipocyte mRNA levels of Foxa-2 of mice that lack the insulin receptor in the liver (LIRKO) and exhibit hepatic insulin resistance was also measured. These

mutant mice had fasting plasma hyperinsulinemia compared to control animals (1.76±0.3 vs. 0.37+0.06 ng/ml, respectively) in the absence of obesity (body weight: 22.8 g ± 0.9 g vs. 23.6 g ± 1.1 g, epidymidal fat pad: 12±0.02 vs 0.12±0.02, $Ir^{lox/lox}$ vs. LIRKO, respectively, LIRKO vs. $Ir^{lox/lox}$ n=5) (Michael et al. (2000) Mol. Cell 6:87-97.) Foxa-2 expression was induced in adipocytes of LIRKO mice but was absent in $Ir^{lox/lox}$ control mice (Fig. 2a). Furthermore, mice with adipocyte-specific insulin resistance due to the ablation of the insulin receptor in fat (FIRKO) were not hyperinsulinemic (0.19±0.02 vs. 0.27 ±0.07, FIRKO vs. $Ir^{lox/lox}$, respectively, n=5) and did not express Foxa-2 in adipose tissue. (Bluher et al. (2002) Developmental Cell 3:25-38.) These findings indicate that Foxa-2 expression in adipocytes correlates primarily with insulin levels and is not induced by tissue insulin resistance or obesity per se.

[0081] Given the strong positive correlation between serum insulin levels and adipocyte Foxa-2 gene expression, the ability of insulin per se to induce Foxa-2 expression was examined.

[0082] Primary adipocytes were isolated from lean wildtype C57B6 mice and cultured in MEM medium (control) or in the presence of insulin (100 nM; 24 hours and 60 hours, respectively) or rosiglitazone (50 μM), WY14,643 (100 μM), dexametason (1 μM), leptin (100 ng/ml), TNF-α(5 ng/ml), adiponectin (500 ng/ml) and glucagons (100 nM) for 60 hours. Gene expression was measured by semiquantitative RT-PCR. Steady state mRNA levels of Hprt were used as a control and indicate that each lane contains similar amounts of mRNA. Reactions were also assayed in the absence of reverse transcriptase, showing that mRNA was not contaminated with genomic DNA.

[0083] As shown in Fig. 2b, a strong induction of Foxa-2 was observed after 60 hours of insulin stimulation.

[0084] De novo expression of Foxa-2 could not be induced by other factors that are known to have potent effects on adipocyte differentiation and

metabolism, including Ppar-α and -γ agonists, glucocorticoids, leptin, TNF-α, adiponectin, IL6, glucagon and high glucose concentrations (20 mmol/L) (Fig. 2b).

[0085] The effect of insulin on adipocyte gene expression of obese (ob/ob) animals was also analyzed.

[0086] Primary adipocytes were isolated from ob/ob mice and cultured in MEM medium (control) or in the presence of insulin (100 µM; 24 hours and 60 hours, respectively) or rosiglitazone (50 µM), WY14,643 (100 µM), dexamethason (1 µM), leptin (100 ng/ml), TNF-α, (5 ng/ml), adiponectin (500 ng/ml) and glucagons (100 nM) for 60 hours. Gene expression was measured by semiquantitative RT-PCR. Steady state mRNA levels of Hprt were used as a control and indicate that each lane contains similar amounts of mRNA. Reactions were also assayed in the absence of reverse transcriptase, showing that mRNA was not contaminated with genomic DNA.

[0087] As shown in Fig. 2c, prolonged culturing of primary adipocytes from these animals led to a strong increase in Foxa-2 expression. Ppar agonists, gluococorticoids, leptin, TNF-α, adiponectin, IL6, glucagons and glucose were unable to raise Foxa-2 expression in ob/ob adipocytes.

Example 4

Foxa-2 inhibits adipocyte differentiation

[0088] The physiological role of Foxa-2 was investigated by generating preadipocyte (3T3-L1) cell lines that express Foxa-1, Foxa-2 or Pref-1.

[0089] Cells were transfected with vector pcDNA3 (control) or expression vectors containing cDNAs of Foxa- 1, Foxa-2 and Pref-1 under the control of a constitutive promoter. After selection with neomycin, pools of stable transfectants were induced with differentiation medium (not containing insulin).

At day 8 post-induction, cells were either stained for lipid accumulation using Oil Red 0 or mRNA and total protein extracts were prepared.

[0090] Expression of Foxa-2 or Pref-1 inhibited adipocytes differentiation in the presence of a pro-differentiation medium (Fig. 3a). In contrast, cells expressing Foxa-1 or the empty expression vector (pcDNA3) were able to accumulate lipid droplets (Fig. 3a).

[0091] Gene expression profiles were measured by RT-PCR. Hprt expression was used as a loading control indicating that each sample contained similar amounts of mRNA. No products were amplified in the absence of reverse transcriptase. Results are shown in Fig. 3b.

[0092] Western blotting was performed on cell extracts from undifferentiated and differentiated 3T3-L1 cell lines. Total protein was separated by SDS-PAGE and analyzed by immunoblotting for Foxa-2 and aP2 expression. TATA binding protein (Tbp) expression was measured as a loading control. Results are shown in Fig. 3c.

[0093] Gene expression was analyzed in a vasculo-stromal fraction (containing preadipocytes) of wildtype and ob/ob mice that were cultured in the absence (—) or presence of 100 nM insulin for 24 and 60 hours. Results are shown in Fig. 3d.

[0094] RT-PCR analysis and immunoblot analysis revealed that Foxa-1 and Foxa-2 were expressed in transfected 3T3-L1 cells but absent in untransfected cells. (Figs. 3b and c).

[0095] Consistent with the morphological differentiation, ectopic expression of Foxa-2 prevented the down-regulation of Pref- 1, Gata-2 and Gata-3, all of which have been shown to inhibit adipocyte differentiation (Fig. 3b). Smas et al. (1993) Cell 73:725-734; Tong et al. (2000) Science 290:134-138. Foxa-2 expression inhibited the induction of late markers of adipocyte

differentiation such as Ppar-y, adipocyte fatty acid binding protein (aP2) and fatty acid synthase (Fas) (Fig. 3b, c).

[0096] To determine whether Foxa-2 is a direct activator of the Pref-1, Gata-2 or Gata-3 genes, the expression of these genes was compared in wt (Foxa-$2^{+/+}$), heterozygous (Foxa-$2^{+/-}$) and null (Foxa-$2^{-/-}$) embryonic stem cells (Duncan et al. (1998) Science 281:692-695). Wt (R1, +/+), heterozygous (B13, 4B1, +/-), and homozygous (B14, 5.1, 5.2, -/-) Foxa-2 ES cells were differentiated into EBs as described by Tong et al. (2000) Science 290:134-138 and assayed for Hprt, Foxa-2, Gata-2, Gata-3, Gata-4 and Pref-1 mRNA expression by RT-PCR. Results are shown in Fig. 3e. No differences in Gata-2 and Gata-3, gene expression were found in EBs of different Foxa-2 genotypes. In contrast, Pref-1 expression was markedly reduced in Foxa-$2^{-/-}$ EBs compared to wt cells.

[0097] The promoter of the Pref-1 gene was analyzed to explore whether the Pref-1 gene is a direct target of Foxa-2.

[0098] The Pref-1 transcription start site was mapped by 5'RACE and a 1.3 kb fragment of 5'-regulatory sequence was cloned into a reporter vector containing the luciferase gene (pPref-Luc). Sequence analysis of the promoter sequences identified two Foxa binding sites at position -621 and -316 that were highly conserved between mouse and human. Expression of Foxa-2 in 3T3-L1 cells in the presence of reporter construct pPref-Luc that contained a 1.3 kb promoter sequence upstream of the luciferase gene revealed a six-fold activation compared to cells that do not express Foxa-2 (Fig. 4c). Deletion of the upstream Foxa element reduced the transcriptional activity by 50%, suggesting that this element is important for Pref-1 gene expression. Constitutive Pref-1 expression markedly inhibits 3T3-L1 adipocyte differentiation (Smas et al. (1993) Cell 73:725-734) and downregulation of Pref-1 expression promotes adipogenesis (Sui et al. (2000) J. Obesity S15-S19). Furthermore, Pref-1 mutant mice have increased fat accumulation compared to wt littermates (Moon et al. (2002) Mol. Cell Biol.

22:5585-5592). Together, these data indicate that Foxa-2 inhibits adipocyte differentiation in by transcriptional activation of the Pref-1 gene.

Example 5

Foxa-2 is an insulin-regulated gene in primary preadipocytes

[0099] Stromal vascular cells from ob/ob mice are known to have increased cell replication in vitro and accumulate little triglycerides when cultured in differentiation medium containing insulin compared to lean control animals (Black et al (1995) J. Cell. Biochem 58:455-463). These data indicate that cells from obese mice are resistant to differentiation under conditions that support extensive differentiation in lean mouse cells. To test if the resistance to differentiation of obese preadipocytes may be mediated by Foxa-2, stromal vascular cells of lean and obese mice were isolated, cultured in the presence or absence of insulin, and gene expression of Foxa-2 and Pref-1 was measured. The mRNA levels were markedly higher in stromal vascular cells of ob/ob mice compared to lean littermates. The expression of Foxa-2 in stromal vascular cells of lean and obese mice could be markedly increased by insulin. However, Foxa-2 induction in ob/ob preadipocytes was profoundly higher compared to wildtype cells. Furthermore, Foxa-2 expression correlated with induction of Pref-1 gene expression. Together, these data indicate that Foxa-2 is an insulin-regulated gene in primary preadipocytes that may counter-regulate adipocyte differentiation under conditions that support extensive differentiation.

Example 6

Foxa-2 is a transcriptional regulator in adipocytes

[00100] Foxa proteins regulate the expression of many metabolic genes through interaction with specific binding sites in promoters/enhancers that lead to chromatin remodeling and transcriptional activation. (Chaya et al. (2001) J. Biol. Chem. 276:44385-44389; Cirillo et al. (1988) EMBO J. 17:244-254.)

[00101] To determine whether Foxa-2 is an important transcriptional regulator in adipocytes, expression analysis of genes that have putative Foxa binding sites in their 5-regulatory sequences was performed in Foxa-2 expressing preadipocytes and in differentiated adipocytes (Fig. 4a). It was found that mRNA levels of the insulin receptor (Ir), insulin receptor substrate-2 (Irs-2), hormone sensitive lipase (Hsl), lipoprotein lipase (Lpl), glucose transporter-4 (Glut-4), muscle isoform of pyruvate kinase (m2Pk), hexokinase-2 (Hk-2) and uncoupling proteins-2/3 (Ucp-2, Ucp-3) were increased in 3T3-L1 cells that expressed Foxa-2 (Fig. 4a). The expression of Ucp-l, mitochondrial ATP-citrate lyase, glycerol 3-phosphate dehydrogenase, acyl-CoA carboxylase, Srebp-1 c, mitochondrial pyruvate decarboxylase, mitochondrial carnitine transporter, and Irs-1 were unaffected by Foxa-2. The observed expression changes were Foxa-2-specific since they are not present in preadipocytes expressing Foxa-1 (Fig. 4a). Differentiation of cells transfected with pcDNA3 (control) or expressing Foxa-1 led to a differentiation-dependent increase in the expression of Ir, Irs-2, Hsl, Lpl, Glut-4, Hk-2, Ucp-2 and Ucp-3. Foxa-2 expression blocked adipocyte differentiation and thereby, prevented the induction of these genes. These observations underline the important effect of the status of adipocyte differentiation state on gene expression. It was found that steady state mRNA levels of mPk were not affected by the differentiation state of 3T3-L1 cells and expression levels remained markedly increased in Foxa-2 expressing cells. This indicates that Foxa-2 is also an important activator of metabolic genes whose expression is not affected by adipocyte differentiation per se.

[00102] Expression levels of these genes in adipocytes of ob/ob mice and lean littermate controls were measured by RT-PCR. Results are shown in Fig. 4b.

[00103] Each lane indicates a different animal. Semi-quantitative measurements of gene expression were obtained by densitometry, and ob/ob/wt indicates the ratio of adipocyte mRNA expression levels of the means of wt and ob/ob mice. The levels of significance of the comparison wt vs. ob/ob are shown on the right.

[00104] A striking correlation was observed between Foxa-2 expressing adipocytes of ob/ob animals and increased expression of putative Foxa-2 target genes, including Pref-l, Hk-2, m2Pk, Glut-4 and Ucp-2/3 (Fig. 4b). Moreover, primary adipocytes that were cultured in the presence of 100 nM insulin for 60 hours to induce Foxa-2 expression also showed an upregulation of these genes compared to untreated adipocytes (Fig. 4e). Gene expression was measured by semiquantitative PCR. Experiments were carried out in triplicate.

[00105] To determine whether Foxa-2 can directly activate the above-mentioned genes, the promoters were characterized. The promoters of Ucp-2, Lpl, Hk-2 and Pref-1 were cloned upstream of a luciferase reporter gene. Transcriptional activation was assayed in the absence (pcDNA3) and presence of Foxa-1 and Foxa-2 by transfecting 3T3-L1 cells with the expression vectors indicated in Fig. 4c, pCMB-β-Gal, and the luciferase reporter constructs. Luciferase activity was normalized to β-Gal activity. Each value in Fig. 4c represents the mean of 9 independent experiments $\pm$SD. As shown in Fig. 4c, Foxa-2 transactivates the promoters of Ucp-2, Lpl, Pref-1 and Hk-2 in 3T3-L1 cells.

[00106] A dose-dependent activation of all promoters was detected when co-transfected with a plasmid expressing Foxa-2. Transactivation of the reporter gene was completely lost when the Foxa binding site in the Ucp-2 promoter was

selectively mutated, indicating that the Foxa binding site in the Ucp-2 promoter is functionally important (Fig. 4c).

[00107] Electrophoretic mobility shift assays were performed to determine whether Foxa-2 can bind to the putative binding sites in Ucp-2, Lpl, Hk-2 and Pref-1 promoters.

[00108] ^{32}P-labeled probes corresponding to putative Foxa-2 binding sites in the promoters of Pref- 1, Lpl and Ucp-2 were incubated with nuclear extracts from ob/ob fat in the presence of either unlabeled probe, anti-Foxa- 1 or anti-Foxa-2 antibody. (Weinstein et al. (1994) Cell 78:578-588.) Protein/DNA complexes were separated on a 4% acrylamide gel and visualized by autoradiography. Results are shown in Fig. 4d. The radioactive probes (bottom of the gel) are not shown. *: $P<0.01$, **: $P<0.001$. Hprt: hypoxanthine phosphoribosyltransferase, Ir: insulin receptor, Irs-2: insulin receptor substrate, Hsl: hormone-sensitive lipase, Lpl: lipoprotein lipase, mPk: muscle isoform of pyruvate kinase, Hk-2: hexokinase-2, Ucp-2/3: uncoupling protein-2/3.

[00109] A major DNA/protein complex was detected with nuclear extracts from ob/ob but not wt mice (Fig. 4d). This binding activity could be competed by an unlabeled excess of 'cold' Foxa binding oligonucleotides. Furthermore, supershifts of the complexes were detected after preincubation of the complexes with a monospecific Foxa-2 antibody but not with an anti-Foxa-1 antiserum. The supershifts of the DNA/protein complexes were almost complete, suggesting that Foxa-2 is a major forkhead transcription factor that binds to these sites in adipose tissue of ob/ob animals.

Example 7

Development of diet-induced obesity and metabolic analysis of primary adipocytes

[00110] The gene expression data in the foregoing examples indicate that Foxa-2 is a powerful transcriptional activator of genes responsible for glucose uptake (Glut-4) and metabolism (Hk-2, m2Pk), insulin signaling (Ir, Irs-2), lipid metabolism (Hsl) and possibly energy dissipation (Ucp-2, Ucp-3) that can be predicted to influence adipogenesis. (Spiegelman et al. (1993) J. Biol. Chem. 268:6823-6826; Boss et al. (2000) Diabetes 49:143-156; Olefsky (1976) Endocrinology 100:1169-1177; Tozzo et al. (1997) Endocrinology 138:1604-1611.) To test this hypothesis in vivo, mutant Foxa-2 mice that have one inactivated Foxa-2 allele (Foxa-$2^{+/-}$) by targeted insertion of the LacZ gene were studied. (Weinstein et al. (1994) Cell 78:578-588.) Haploinsufficient Foxa-2 mice were studied because Foxa-2 null mice have an early embryonic lethal phenotype (at E7.5) and heterozygous mice exhibit normal glucose and lipid metabolism. (Ang et al. (1994) Cell 78:561-574; Shih et al. (2002) Proc. Natl. Acad Sci, USA 99:3818-3823). Foxa-$2^{+/-}$ mice and wildtype littermates were fed a high fat (55% fat) diet and studied metabolically. RT-PCR and Xgal staining of fat from Foxa-$2^{+/-}$ animals confirmed that these mice lacked Foxa-2 in adipocytes at the beginning of the study but induced expression during seven weeks of high fat diet (Fig. 2a, 5a, b). Fasting blood glucose, insulin, TNF-α, free fatty acid and triglyceride levels were similar between Foxa-$2^{+/-}$ and control animals (Table 1). Mice on a high fat diet increased their fasting plasma insulin levels approximately four-fold compared to animals on a chow diet. Foxa-$2^{+/-}$ mice exhibited a markedly increased weight gain compared to control mice when kept on a high fat diet, in spite of similar food intake and physical activity (10 g. vs. 6 g. after 42 days of high fat diet, respectively) (Fig. 5c, d). Resting heat and CO_2 production was diminished in Foxa-$2^{+/-}$ mice, indicating that they are hypometabolic (Fig. 5e,

f). Foxa-$2^{+/-}$ animals developed noticeably increased pericardial, intrapentoneal and subcutaneous fat deposits compared to littermate animals. The adipose mass of the epidymidal fat pad was approximately double in Foxa-$2^{+/-}$ mice compared to littermate controls after a seven-week high fat diet (Fig. 5g). The increase in adipocyte mass in Foxa-$2^{+/-}$ mice was due to an increase in fat cell number, the size distribution of adipocytes was similar between mutant and wildtype animals. To test if adipocytes of Foxa-$2^{+/-}$ mice have altered glucose metabolism, [U-^{14}C] glucose uptake and metabolism into lactate, CO_2, lipid glycerol and fatty acids was studied. Glucose uptake and glucose incorporation into CO_2, lactate, and glyceride glycerol was strikingly reduced in adipocytes in Foxa-$2^{+/-}$ mice compared to wildtype littermates (Fig. 5 h-k). In contrast, no differences in adipocyte metabolism were observed in mutant and wildtype adipocytes of lean mice on a normal chow diet. Adipocytes of Foxa-$2^{+/-}$ mice did not exhibit a significant reduction of glucose incorporation into fatty acids, a finding that is consistent with similar expression of genes of the fatty acid synthesis in Foxa-$2^{+/-}$ and control adipocytes (Fig. 5l). However, reduced glycerol release from adipocytes of Foxa-$2^{+/-}$ mice after maximal stimulation with isoproterenol and following inhibition with insulin was observed, suggesting that lipolysis is decreased in adipocytes of Foxa-$2^{+/-}$ compared to control littermates (Fig. 5m). The defect in metabolism in Foxa-$2^{+/-}$ adipocytes correlated with reduced expression of Foxa-2 target genes. Steady state mRNA levels of Foxa-2, Glut-4, Hk-2, m2Pk, Irs-2, Ucp-2 and Ucp-3 were reduced ~50% and more in fat cells of diet-induced obese Foxa-$2^{+/-}$ compared to wildtype mice (Fig. 5n). Together, these data demonstrate that Foxa-2 is an important metabolic regulator of glucose metabolism and energy dissipation in adipocytes of hyperinsulinemic obese mice.

[00111] Figs. 5a and b show X-gal staining of adipose tissue of wildtype (a) and Foxa-$2^{+/-}$(b) mice after a 7-week high fat diet. Figs. 5c-g show relative weight gain (c), food and water intake (d), heat production (e), resting CO_2

production (f), and epidymidal fat pad weight (g) of Foxa-$2^{+/-}$ and wildtype littermates on chow and high fat diets. Figs 5h-l show glucose metabolism into different pathways at 10 and 100 nM insulin in isolated adipocytes from Foxa-$2^{+/-}$ (red) and wildtype (black) littermates. [U-^{14}C]Glucose uptake in isolated adipocytes from epidymidal fat (h), [U-^{14}C]Glucose incorporation into CO_2 (1), lactate (j), lipid glycerol (k), and fatty acids (1), measured after 2 hours incubation in the absence (control) or presence of insulin. Fig. 5m shows glycerol release from adipocytes in the presence or absence of insulin after stimulation of lipolysis with isoperenterol (Isop). Fig. 5n shows measurements of relative gene expression levels of metabolic genes in adipocytes of Foxa-$2^{+/-}$ and wildtype littermates (100%) using semiquantitative RT-PCR. All mice were female, 15 weeks of age, n=4, means ± SD. *: $P \leq 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, *****: $P \leq 1 \times 10^{-5}$, ns: not significant.

[00112] Table 1 below shows plasma levels of insulin, glucose, cholesterol, free fatty acid (FFA), triglycerides (TG), leptin and TNF-α of lean wildtype (C57) and mutant Foxa-2 (Foxa-$2^{+/-}$) mice on a normal (ND) or high fat diet (HF). Data are from n=5 in each group, age: 8 weeks old female mice, duration of diet: 42 days.

Table 1

	Insulin ng/ml	Glucose mg/dl	TNFα pg/ml	Cholesterol mgl/dl	Leptin Eq/L	FAA mmol/L	TG mg/dl
C57	0.42±0.02	96±35	< 10	43±5	0.6±0.1	0.15±0.02	52±5
C57, HF	1.63±0.08	96±24	12.9±0.8	66±5	0.6±0.1	0.16±0.04	62±11
Foxa-2$^{+/-}$	0.50±0.06	84±18	< 10	38±11	0.8±0.1	0.18±0.04	54±11
Foxa-2$^{+/-}$, HF	1.52±0.03	87±8	12.3±1.1	54.9	0.8±0.1	0.21±0.08	80±14

Example 8

Expression of Fxr-1 is positively regulated by Foxa-2

[00113] Expression of murine Fxr-1 and Fxr-2 in differentiated ES cells (embryoid bodies, Ebs) deficient for Foxa-2 was assessed. Wildtype (+/+), heterozygous (+/-) and null (-/-) Foxa-2 ES cells were differentiated and expression levels were analyzed for Gata-4 (a marker for visceral endoderm), Fxr-1 and Fxr-2. As shown in Fig. 6, Fxr-1 expression is absent in cells lacking Foxa-2 expression.

[00114] Murine Fxr-1 and human Fxr promoters were analyzed as follows. HepG2 cells were transfected with vectors expressing the transcription factors indicated in Fig. 7 and with a reporter construct in which the mouse (mFxr-1 or mFxr-2) or human (FXR) promoters are upstream of the luciferase gene. Constructs were cotransfected with CMV-Xgal vector to normalize transfection efficiencies. Luciferase activity was measured 48 hours after transfection. A conserved Foxa binding site was identified in the mouse Fxr-1 and human FXR promoters. As shown in Fig. 2, this promoter can be activated when coexpressed with Foxa-2.

[00115] Electrophoretic mobility shift analysis (EMSA) of the Foxa-binding site in the Fxr-1 promoter was performed. As shown in Fig. 8, lanes 1-5, gel shift analysis with a putative HNF-4 binding site in the Fxr-1 promoter

exhibited no binding. As shown in Fig. 8, lanes 6-11, EMSA with a putative Foxa binding site in the Fxr-1 promoter showed that Foxa-2 protein binds to a consensus sequence in the murine Fxr-1 promoter.

[00116] It was also demonstrated that, like Foxa-2, Fxr is expressed de novo in adipocytes of obese (hyperinsulinemic) mice. Fig. 9a shows de novo expression of Fxr-1 in adipose tissue of ob/ob and db/db mice.

[00117] It was further demonstrated that Fxr expression can be induced by culturing primary adipocytes of lean mice in the presence of insulin (50nM) for 60 hours. Expression of Shp, a known target gene of Fxr, is also induced in insulin treated cells. Results are shown in Fig. 9b.

[00118] These data indicate that expression of Foxa-2 in adipocytes of obese animals activates Fxr and Shp expression.

ABSTRACT OF THE DISCLOSURE

The present invention relates to methods for inhibiting adipogenesis and methods for treating obesity, metabolic syndrome and non-insulin dependent diabetes mellitus by administering an agent that increases Foxa-2 or Fxr, or an agent that activates Fxr. The invention is further related to methods for identifying agents that increase Foxa-2 or Fxr, or activate Fxr, and the use of such agents for treatment of obesity, metabolic syndrome and non-insulin dependent diabetes mellitus.

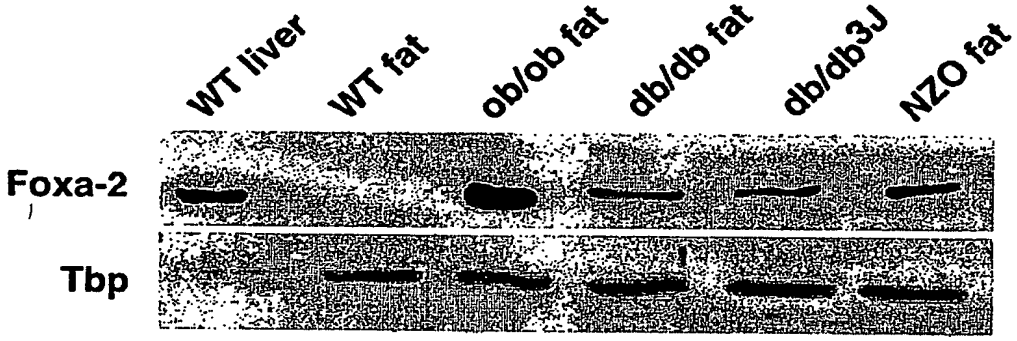
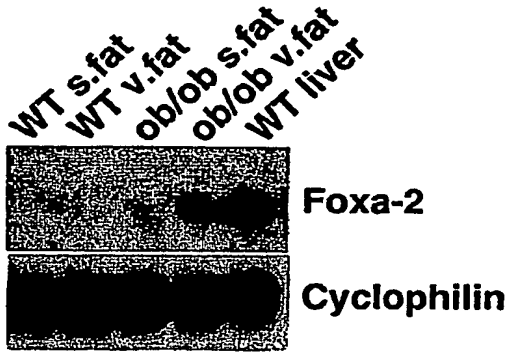
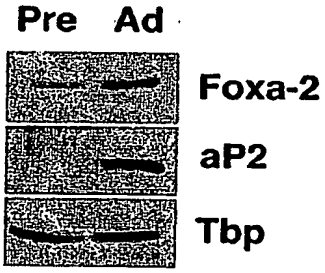


Fig. 1a

b.



c.



Figs. 1b, c

Figs. 1d-f

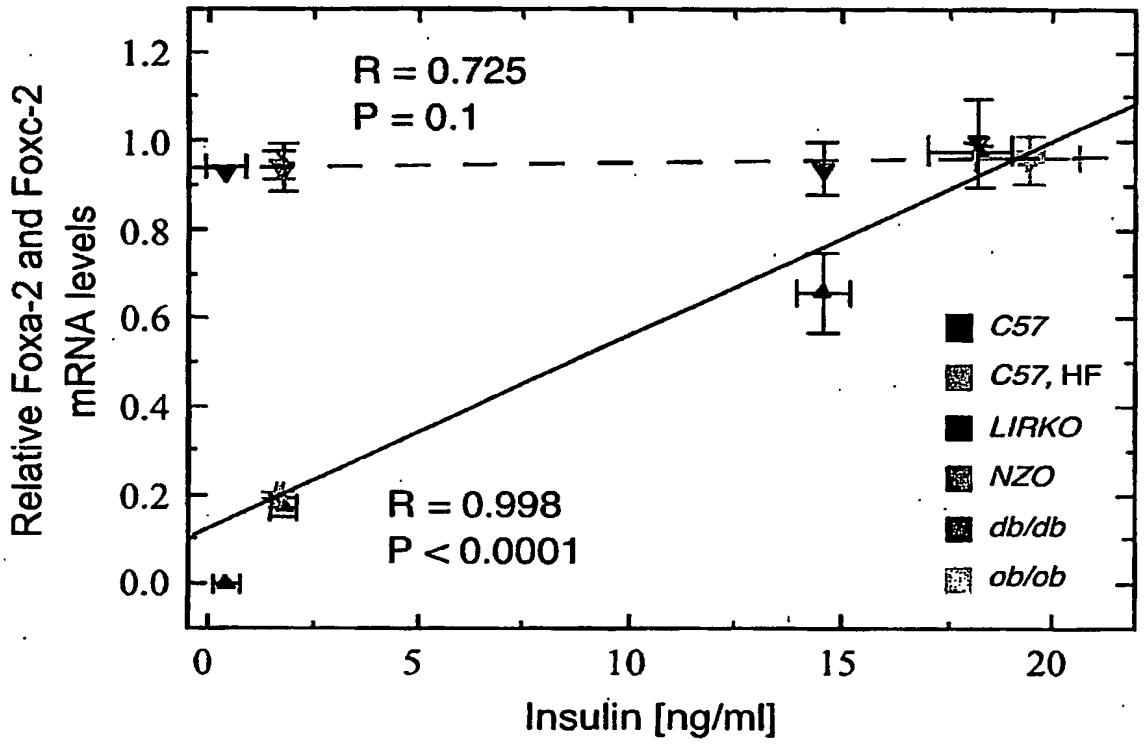

R = 0.725
P = 0.1
R = 0.998
P < 0.0001
C57
C57, HF
LIRKO
NZO
db/db
ob/ob
Relative Foxa-2 and Foxc-2 mRNA levels
Insulin [ng/ml]
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
20

Fig. 2a

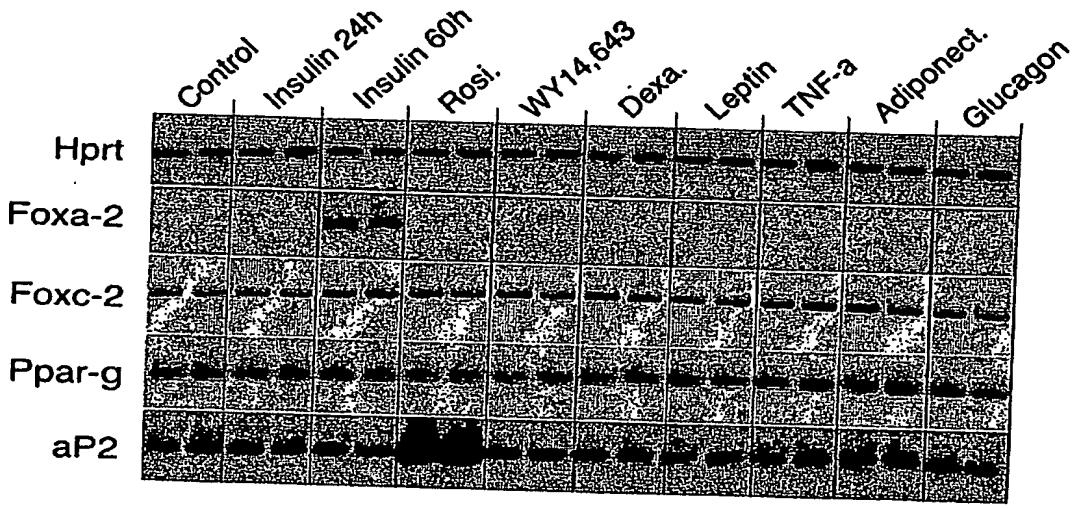


Fig. 2b

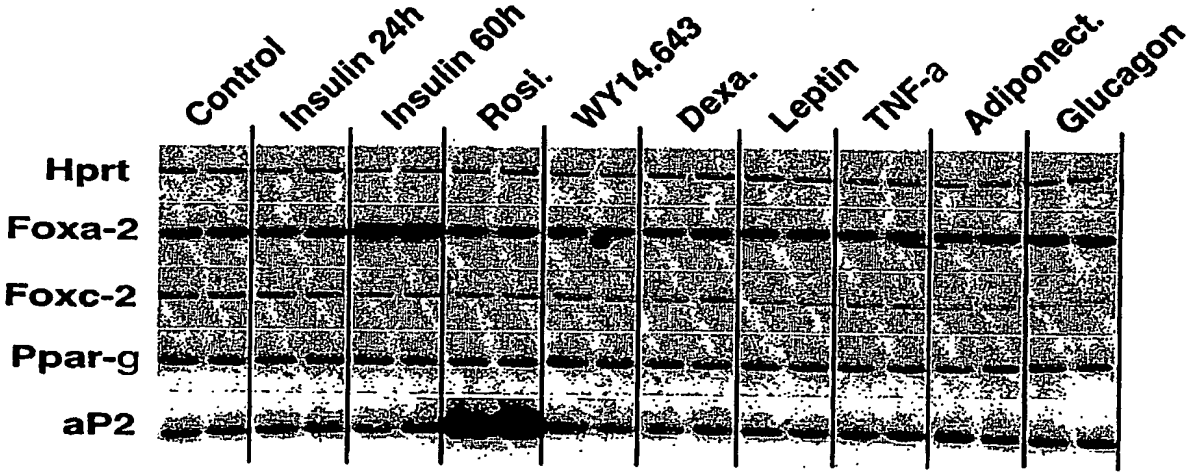
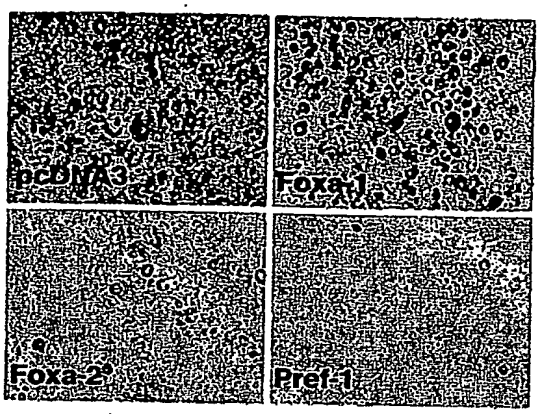
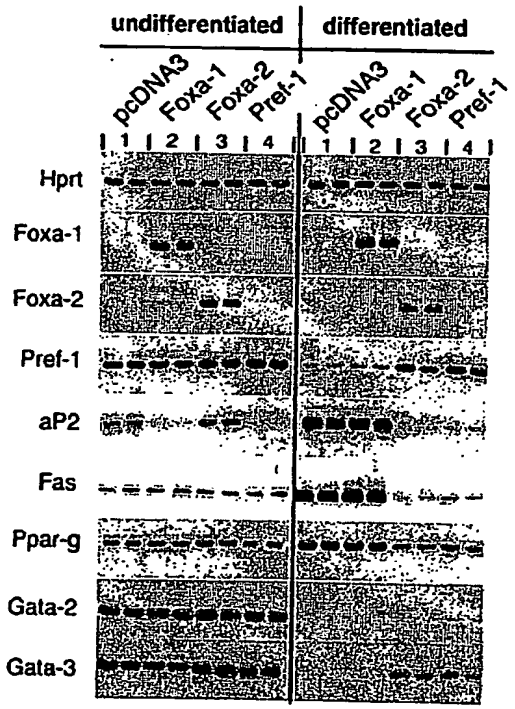


Fig. 2c

a.

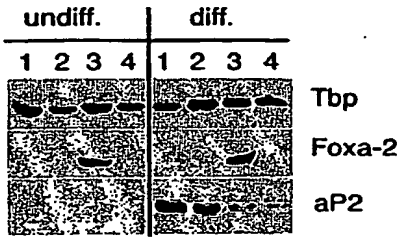


b.

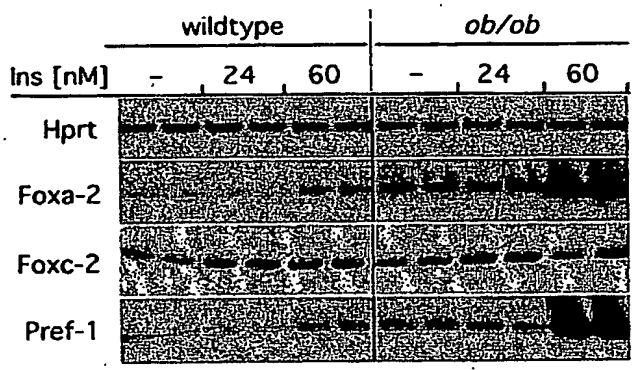


Figs. 3a, b

c.



d.



Figs. 3c, d

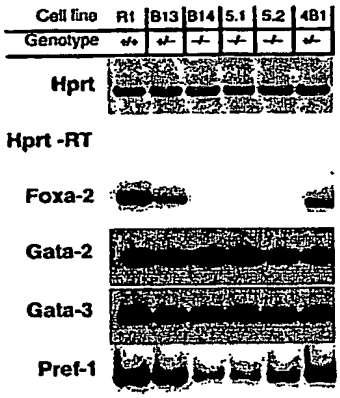


Fig. 3e

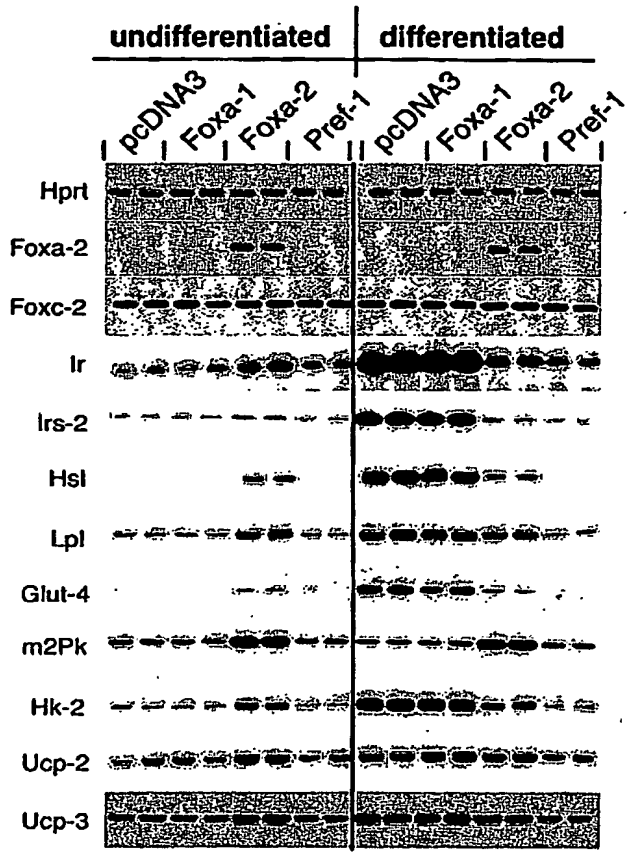


Fig. 4a

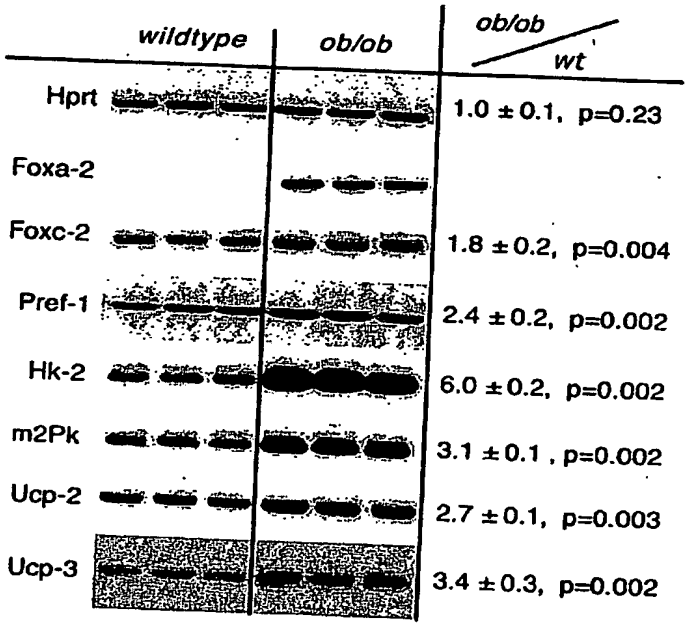


Fig. 4b

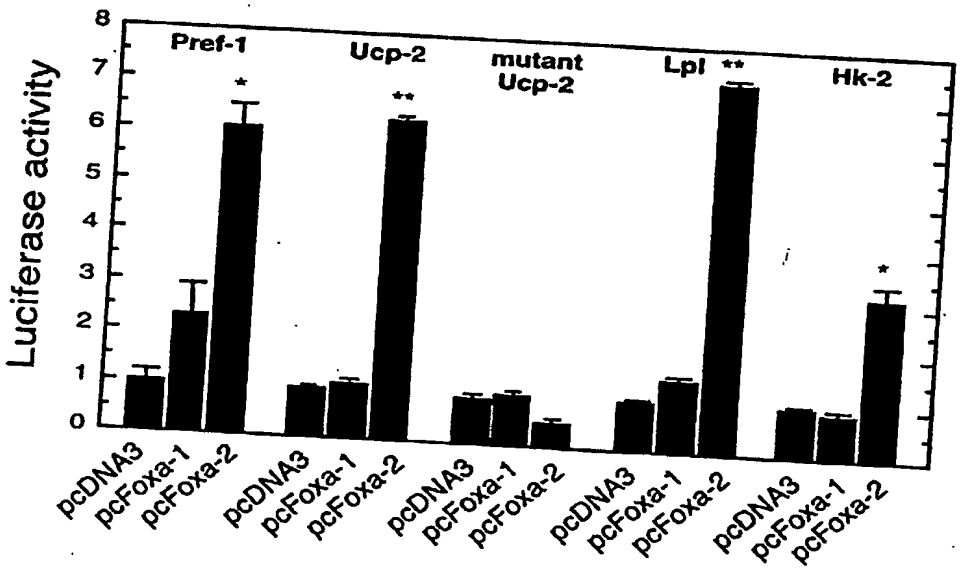


Fig. 4c

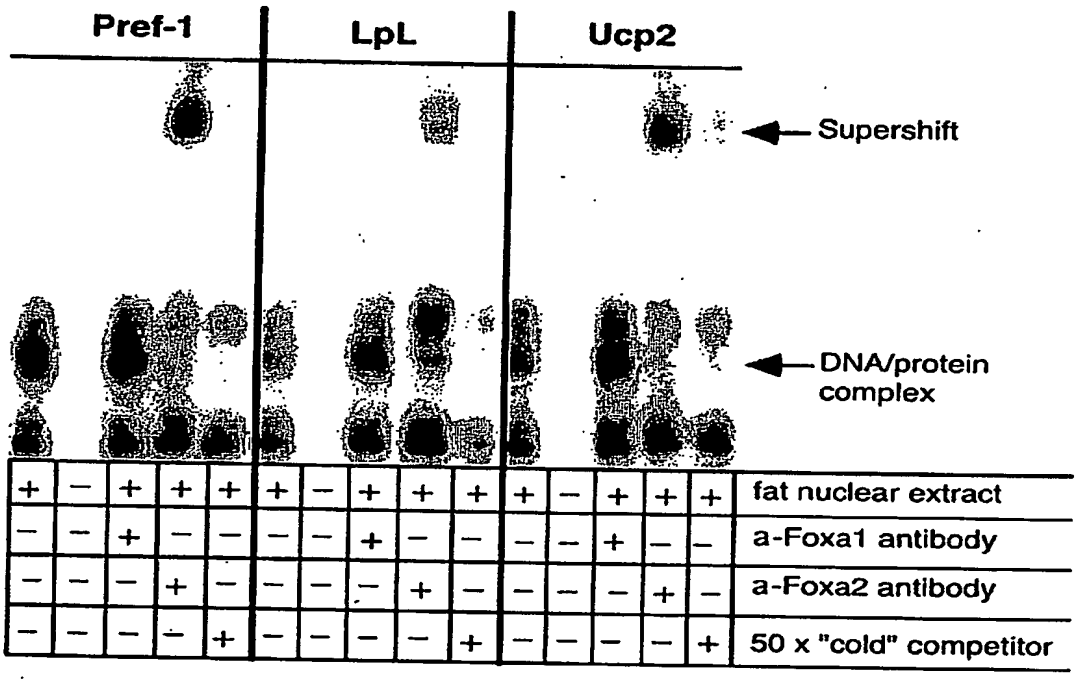


Fig. 4d

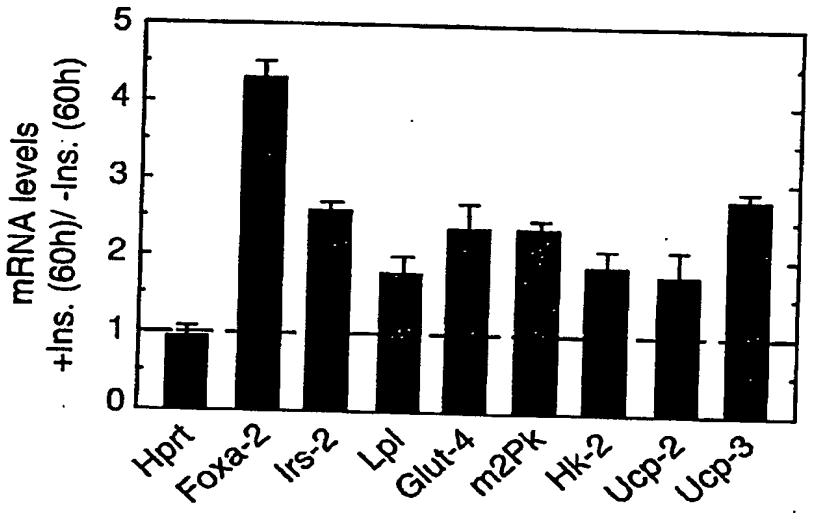
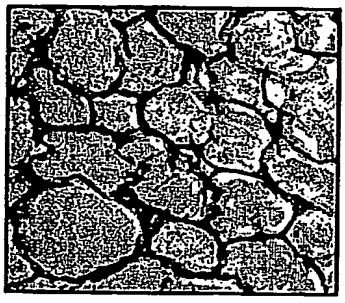


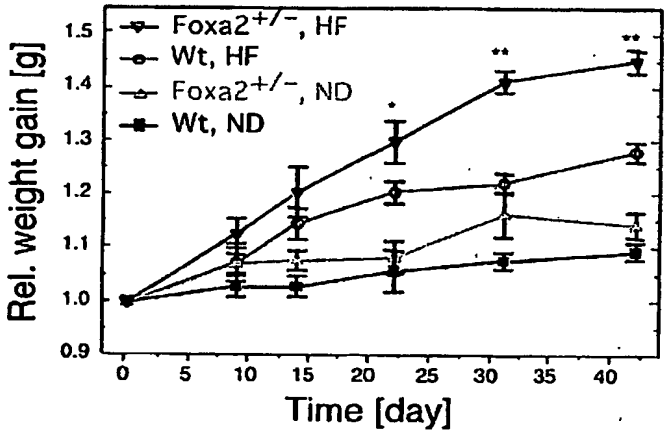
Fig. 4e

a.

b.

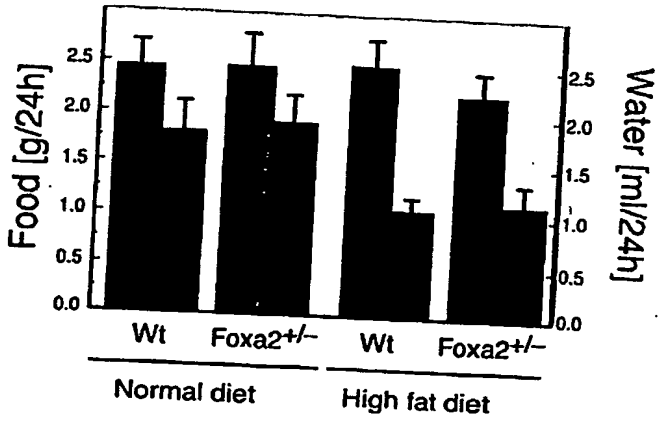


c.

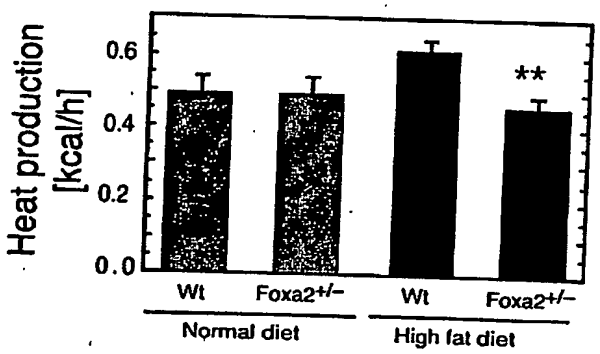


Figs. 5a-c

d.

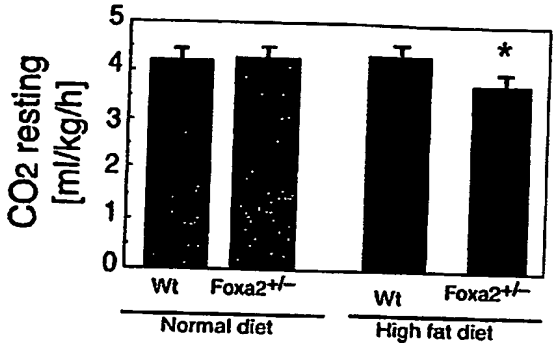


e.

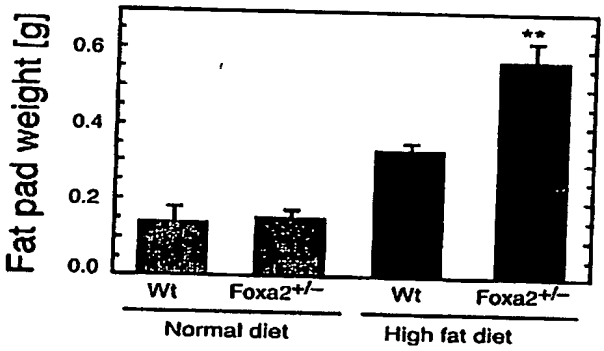


Figs. 5d, e

f.

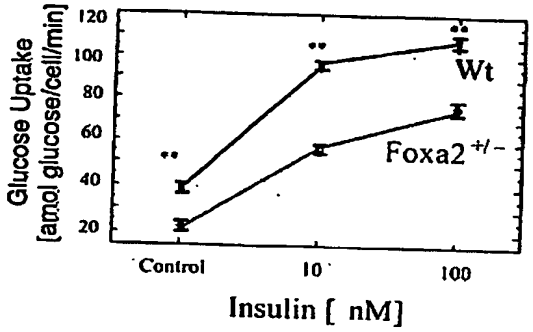


g.

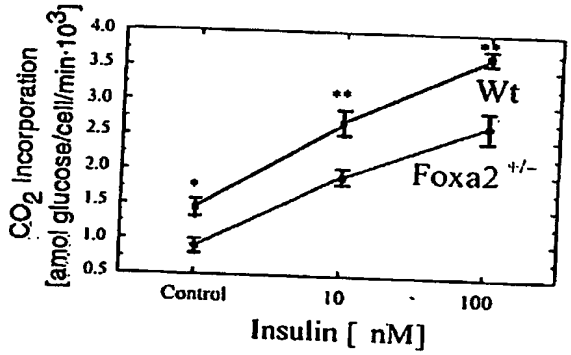


Figs. 5f, g

h.

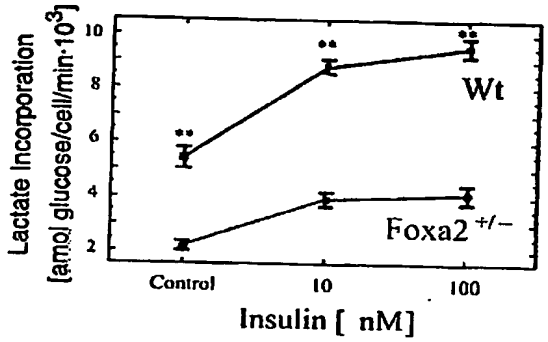


i.

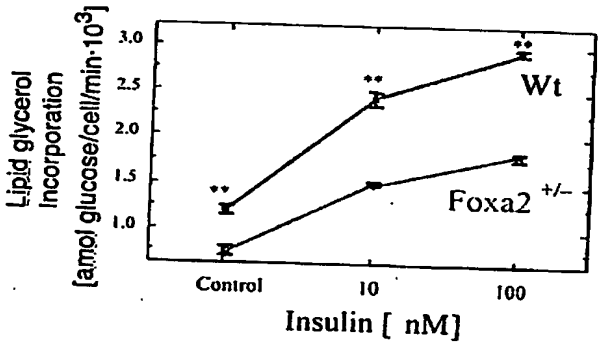


Figs. 5h, i

j.

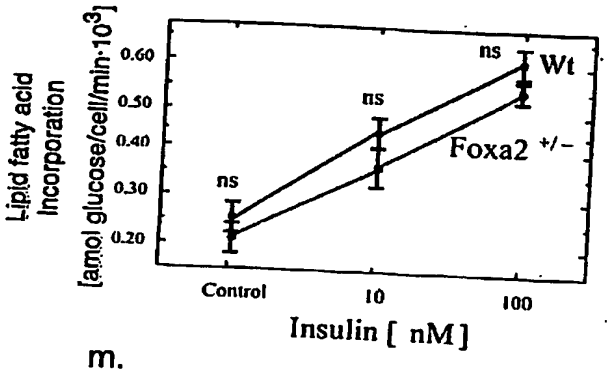

Lactate Incorporation
[amol glucose/cell/min·10³]
10
8
6
4
2
Wt
Foxa2 +/−
Control
10
100
Insulin [nM]

k.

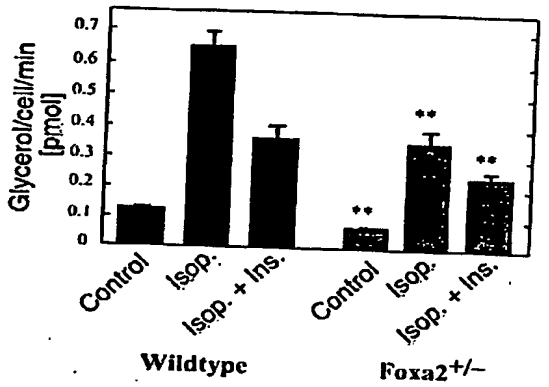

Lipid glycerol
Incorporation
[amol glucose/cell/min·10³]
3.0
2.5
2.0
1.5
1.0
Wt
Foxa2 +/−
Control
10
100
Insulin [nM]

Figs. 5j, k

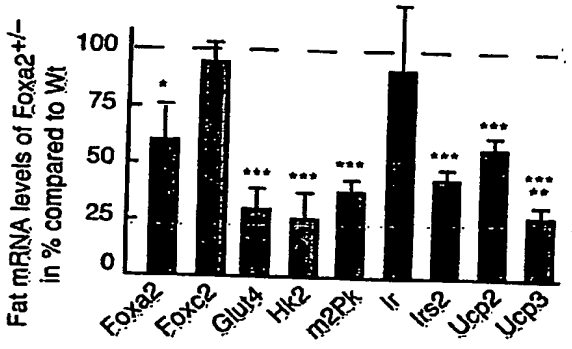
l.



m.



n.



Figs. 5l-n

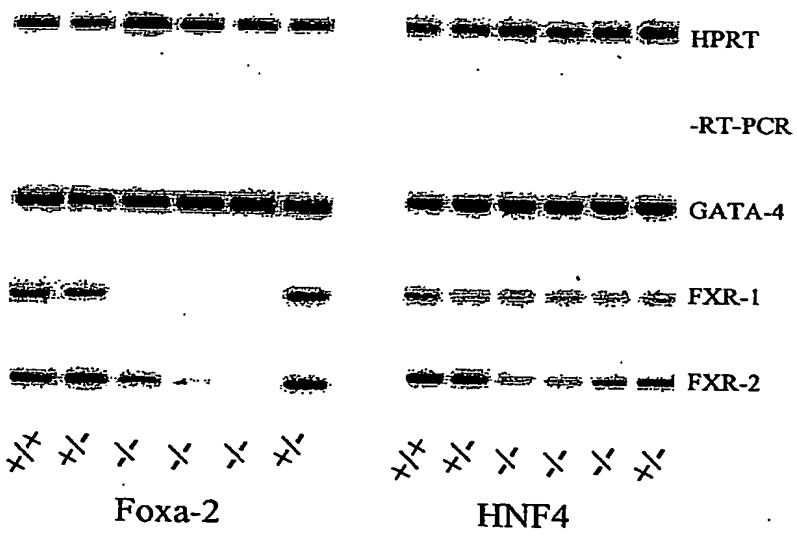


Fig. 6

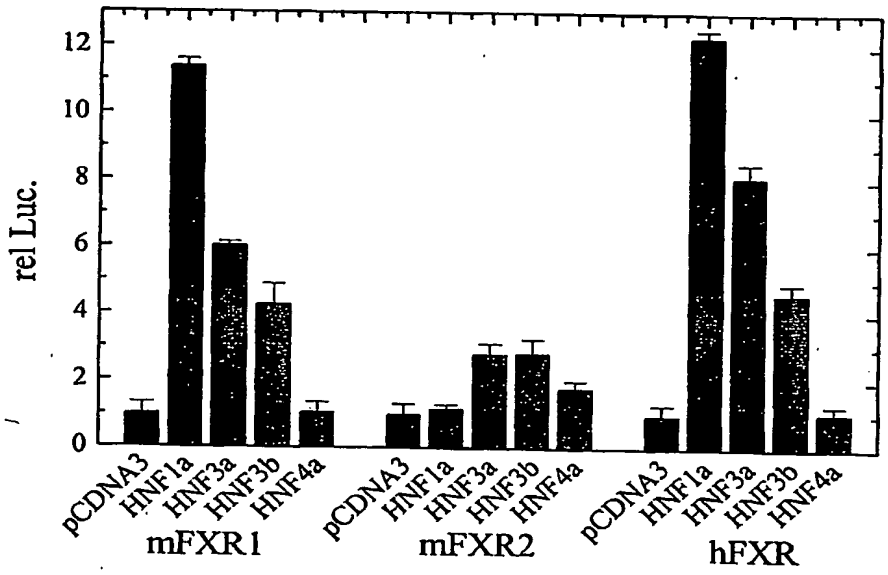
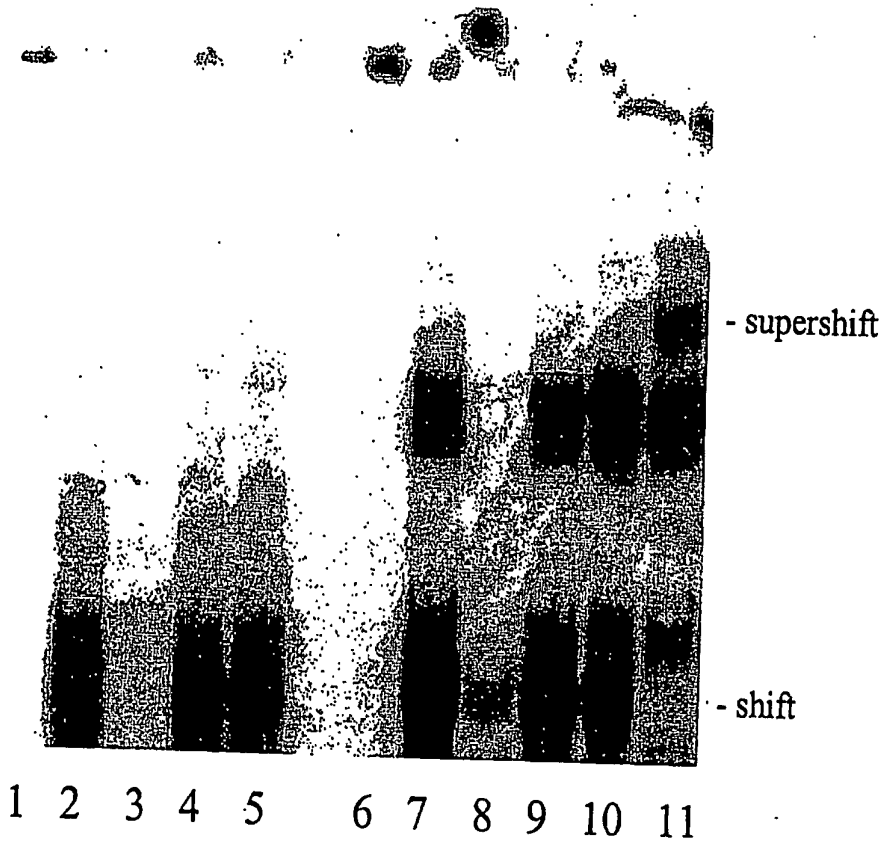


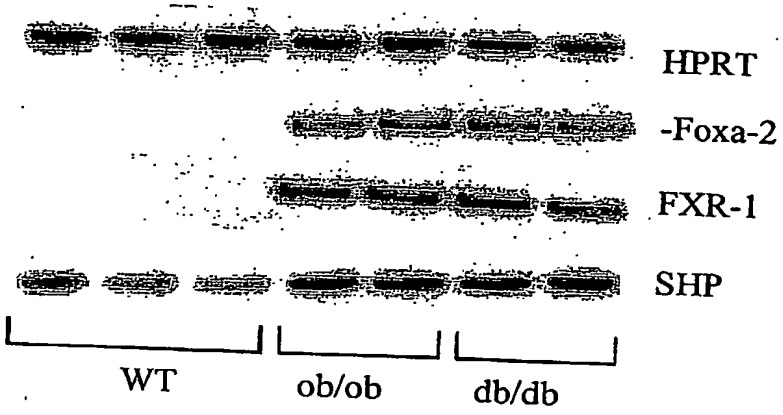
Fig. 7



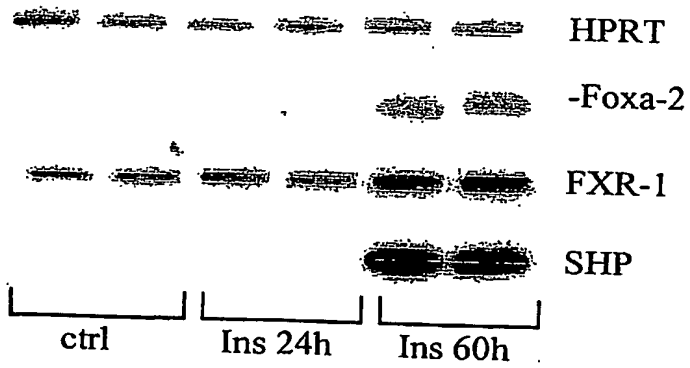
1 probe alone
2 probe + nuc. extract
3 competition WT
4 competition MT
5 supershift
6 probe alone
7 probe + nuc. extract
8 competition WT
9 competition MT
10 competition MT IBAT
11 supershift

Fig. 8

A



B



Figs 9a, b